The phylogeography of an alpine leaf beetle: Divergence within *Oreina elongata* spans several ice ages

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A B S T R A C T

The genetic landscape of the European flora and fauna was shaped by the ebb and flow of populations with the shifting ice during Quaternary climate cycles. While this has been well demonstrated for lowland species, less is known about high altitude taxa. Here we analyze the phylogeography of the leaf beetle *Oreina elongata* from 20 populations across the Alps and Apennines. Three mitochondrial and one nuclear region were sequenced in 64 individuals. Within an mtDNA phylogeny, three of seven subspecies are monophyletic. The species is chemically defended and aposematic, with green and blue forms showing geographic variation and unexpected within-population polymorphism. These warning colors show pronounced east–west geographical structure in distribution, but the phylogeography suggests repeated origin and loss. Basal clades come from the central Alps. Ancestors of other clades probably survived across northern Italy and the northern Adriatic, before separation of eastern, southern and western populations and rapid spread through the western Alps. After reviewing calibrated gene-specific substitution rates in the literature, we use partitioned Bayesian coalescent analysis to date our phylogeography. The major clades diverged long before the last glacial maximum, suggesting that *O. elongata* persisted many glacial cycles within or at the edges of the Alps and Apennines. When analyzing additional barcoding pairwise distances, we find strong evidence to consider *O. elongata* as a species complex rather than a single species.

1. Introduction

The Quaternary has proved a turbulent time for the flora and fauna of northern Europe. This period of Earth's history, from around 2.6 million years ago, has witnessed alternating glacial and interglacial periods driven by interactions between tectonic and orbital forces (Webb and Bartlein, 1992; Williams et al., 1998; EPICA community members, 2004). The fluctuating climate would have had profound effects on population migration and survival, the results of which are still apparent in the community composition and genetic diversity of recent organisms (Bennett, 1990). During glacial periods, when large areas of northern Europe were covered by the growing ice shelf and permafrost, most lowland species survived in southern refugia in the Iberian, Italian and Balkan Peninsulas (Hewitt, 1996, 2000, 2004; Taberlet et al., 1998). Recolonization of temperate regions was affected by mountain ranges such as the Pyrenees or the Alps acting as barriers to some species, so that the contributions of different refugia varied in different taxa. For example, all northern European populations of *Chorthippus parallelus* and *Alnus glutinosa* emerged from the Balkans, whereas in *Erinaceus* and *Quercus* species, western, central and eastern regions were colonized independently from the Iberian, Italian and Balkan refugia, respectively (Hewitt, 1999). This periodic extinction and recolonization shaped the genetic landscape of Europe, determining the large-scale patterns of spatial genetic structure and diversity (Hewitt, 1996, 2001; Gratton et al., 2008; Fussi et al., 2010).

Whilst these patterns have been well documented for many lowland species, much less is known about the influence of ice ages on high altitude animals (Pauls et al., 2006; Schmitt et al., 2006; Haubrich and Schmitt, 2007; Schmitt, 2009). They provide a contrast with the lowland taxa, since the current warm climate represents a period in which their range has probably contracted. Climate cycles may therefore have led currently isolated populations to have come repeatedly into contact during cold periods as their suitable habitat expanded. For the alpine flora, long-term refugia within the Alps and at their northern, southern and eastern

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borders have been proposed on the basis of recent molecular studies (Stehlik et al., 2001; Stehlik, 2002; Schönswetter et al., 2005). Such results have important implications for the community ecology of montane habitats. If many animal and plant species survived *in situ* this suggests a long period for coevolution and local adaptation, which would not have been available to most of the more recently assembled communities of the northern lowlands.

Here we address the bias towards lowland taxa by presenting the phylogeography of the alpine leaf beetle *Oreina elongata* (Suffrian, 1851) (Coleoptera: Chrysomelidae). This species is adapted to survival at high altitudes, with isolated populations found across the Alps and Apennines at altitudes of 1200–2500 m above sea level (Margraf et al., 2003, 2007; Röder et al., 2008). Seven allopatric subspecies have been described based on differentiation of male genitalia (the aedeagus) and cuticle microstructure (Ruffo, 1946; Franz, 1949; Daccordi and Ruffo, 1976, 1986). These herbivorous beetles feed on hosts from two tribes of the Asteraceae: when feeding on *Cirsium* (Cynareae) larvae and adults synthesize cardenolides, whereas individuals feeding on *Adenostyles* or *Senecio* (Senecioneae) encounter plant-produced pyrrolizidine alkaloid *N*-oxides (PAs) that they are able to sequester (Dobler et al., 1996; Hsiao and Pasteels, 1999; Röder et al., 2007; Verdon et al., 2007). This chemical defense is accompanied by what appears to be warning coloration in bright metallic patterns, with blue, green and mixed populations known. Color pattern does not covary with the type of defense, and the within-population polymorphism is unexpected, because learning by predators would be expected to generate positive frequency-dependent selection and lead to monomorphism (Mallet and Joron, 1999).

In the present study we analyzed the genetic structure of 20 populations of *O. elongata* from across the whole species distribution. Sequencing of regions of three mitochondrial genes and one nuclear gene was used to answer the following questions:

1. Did *O. elongata*, as a representative of the high altitude fauna, survive the cold periods of the Quaternary *in situ* in the Alps and Apennines?
2. What does the phylogeography suggest about the evolution of color pattern in the species?

In order to infer the timescale over which differentiation is likely to have arisen, we make use of an approximate molecular dating method based on a review of published gene-specific mtDNA substitution rates to answer a further question:

3. Was divergence within *O. elongata* a product of the last glacial cycle or is the differentiation more ancient?

2. Materials and methods

2.1. Sampling

Between 2001 and 2007, *O. elongata* were collected from 20 populations covering the whole distribution (Alps and Apennines), including most of the sites where the species is known to exist, and all of the seven described subspecies (Ruffo, 1946; Franz, 1949; Daccordi and Ruffo, 1976, 1986; Kippenberg, 1994) (Fig. 1 and Table 1). Samples were preserved in pure ethanol and stored at −20 °C, apart from the individuals from GLE that were dried specimens from a collection. For most populations, three individuals were chosen for the phylogeographic analysis, using only males to be sure of accurate identification based on genitalia (except for PDC, the holotype location for *O. elongata zoiai*, for which only

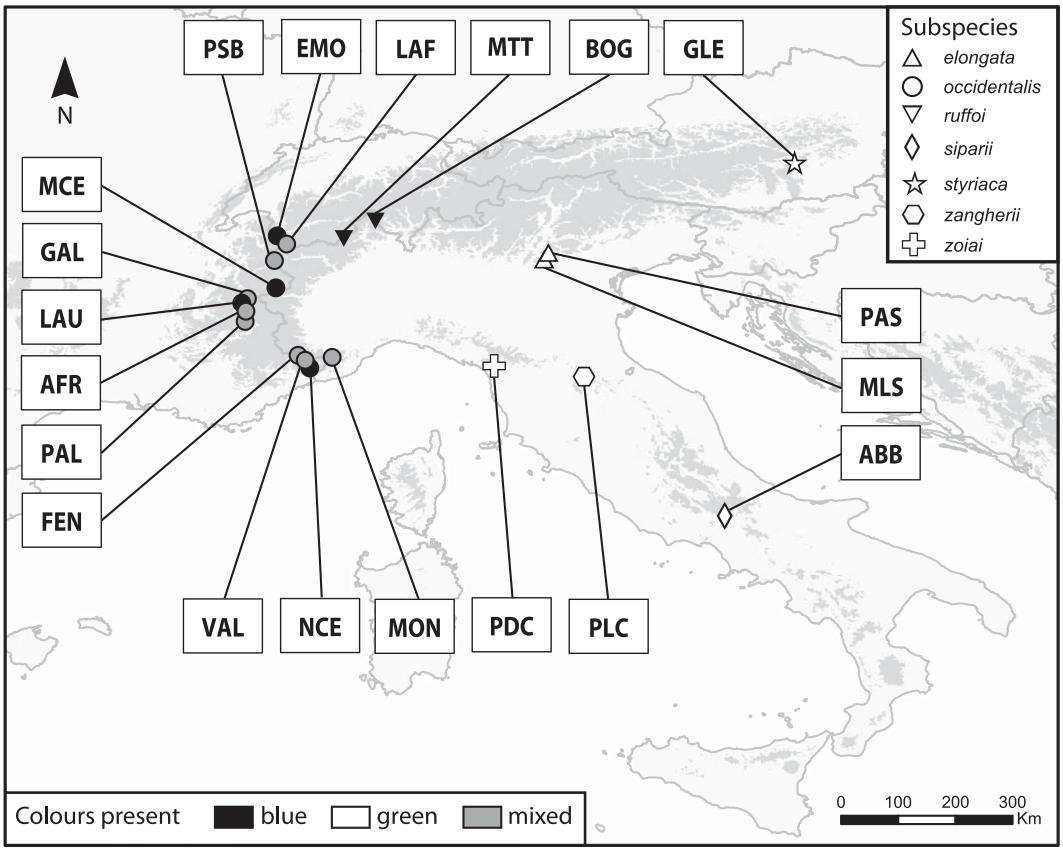

Fig. 1. Distribution of the 20 sampled populations of *O. elongata* in Italy and neighboring countries, showing their subspecies (symbols) and color morphs present (shading). Topographic shading in the Alps and Apennines shows altitudes above 1000 m.

Table 1
Sampled populations of *Oreina elongata* with their geographical coordinates, subspecies, number of treated individuals and year of collection.

Code	Population	Altitude (m a.s.l.)	Geographical coordinates	Subspecies	Sample size	Year
LAF	La Fouly (CH)	2184	45°56′N, 07°04′E	*occidentalis*	3	2006
EMO	Lac Emosson (CH)	1970	46°03′N, 06°55′E	*occidentalis*	3	2007
PSB	Col du Petit St. Bernard (F)	2188	45°40′N, 06°52′E	*occidentalis*	3	2001
MCE	Mont Cenis (F)	2085	45°15′N, 06°54′E	*occidentalis*	6	2005
GAL	Col du Galibier (F)	1999	45°05′N, 06°26′E	*occidentalis*	3	2001
LAU	Col du Lautaret (F)	1811	45°00′N, 06°22′E	*occidentalis*	3	2001
AFR	Ailefroide (F)	1800	44°53′N, 06°26′E	*occidentalis*	4	2001
PAL	Lac Palluel (F)	2479	44°43′N, 06°24′E	*occidentalis*	3	2001
FEN	Col de Fenestre (F)	2470	44°06′N, 07°21′E	*occidentalis*	3	2007
VAL	Terme di Valdieri (I)	2340	44°12′N, 07°15′E	*occidentalis*	3	2001
NCE	L'Authion (F)	2080	44°00′N, 07°26′E	*occidentalis*	3	2001
MON	Monte Mongioie (I)	1920	44°10′N, 07°47′E	*occidentalis*	3	2001
PDC	Pania della Croce (I)	1718	44°02′N, 10°19′E	*zoiai*	3	2007
PLC	Passo la Calla (I)	1260	43°51′N, 11°44′E	*zangherii*	3	2007
ABB	Pizzone (I)	–	41°41′N, 13°57′E	*siparii*	3	2001
MLS	Giazza (I)	1510	45°41′N, 11°06′E	*elongata*	3	2001
PAS	Monte Pasubio (I)	2110	45°47′N, 11°10′E	*elongata*	2	2001
GLE	Glein (A)	1570	47°13′N, 15°03′E	*styriaca*	4	2004
BOG	Bosco Gurin (CH)	1835	46°18′N, 08°27′E	*ruffoi*	3	2001
MTT	Mattmark (CH)	2239	46°01′N, 07°58′E	*ruffoi*	3	2001

A, Austria; CH, Switzerland; F, France; I, Italy; m a.s.l., metres above sea level.

larvae could be obtained). Trees were rooted using the three most closely related species (Hsiao and Pasteels, 1999): *Oreina virgulata*, *Oreina cacaliae* and *Oreina speciosissima*.

2.2. Molecular methods

Total genomic DNA was extracted from four legs of each individual using the DNeasy Tissue Kit (Qiagen, Hilden, Germany). Three regions of mtDNA and one nuclear region were amplified using universal insect primers: a fragment of 16S ribosomal RNA [modLR-J-12887 (5′-CACCGGTTTGAACTCAGATC-3′) with LR-N-13398 (Simon et al., 1994)]; cytochrome oxydase subunit I (COI) [C1-J-1751 with C1-N-2191 (Simon et al., 1994)]; cytochrome oxidase subunit II (COII) [modTL2-J-3037 with modC2-N-3661 (Mardulyn et al., 1997)]; and part of the nuclear region ITS2 [ITS3 with ITS4 (Gomez-Zurita and Vogler, 2003)]. Fragments were amplified using a standard 30 μl PCR mix including: 3 μl of extracted DNA, 3 μl of 10× PCR buffer (Promega, Madison, USA), 3 μl of $MgCl_2$ solution (25 mM), 3 μl of dNTPs (1.5 mM), 0.5 μl of forward and reverse primer (Microsynth, Balgach, Switzerland), 0.3 μl of Taq DNA polymerase (Promega, Madison, USA), all made up to a final volume of 30 μl with purified MilliQ water. The PCR were run in a Biometra TGradient thermocycler (Biometra, Goettingen, Germany) using the following programs: for 16S and COI, initial denaturation for 1.5 min at 93 °C, 36 cycles (35 s at 93 °C, 1 min at 45 °C, 1.5 min at 72 °C), then final elongation of 8 min at 72 °C; for COII, initial denaturation of 1.5 min at 93 °C, 36 cycles (35 s at 93 °C, 1 min at 53 °C, 2 min at 72 °C), then final elongation of 8 min at 72 °C; for ITS2, initial denaturation of 1.5 min at 93 °C, 36 cycles (35 s at 93 °C, 1 min at 48 °C, 1 min at 72 °C), then final elongation of 8 min at 72 °C. The amplified products were purified using the QIAquick PCR Purification Kit (Qiagen, Hilden, Germany) following the manufacturer's specifications. Sequencing by Macrogen Inc. (Seoul, South Korea) was carried out with both forward and reverse primers under BigDye™ terminator cycling conditions, purifying the products using ethanol precipitation and running them using an Automatic Sequencer 3730xl (Applied Biosystems, Foster City, USA).

2.3. Sequence alignment

Sequences (forward and reverse) were assembled and manually corrected using the software Chromas Pro version 1.34 (Technelysium, Helensvale, Australia). The protein coding nucleotide sequences of COI and COII were checked for reading frame errors and termination codons in Mega 4 (Tamura et al., 2007). Alignment was carried out using ClustalW Multiple Alignment (Thompson et al., 1994) within the software Bioedit version 7.0.5.3, followed by minor manual correction.

2.4. Phylogenetic analysis and divergence time estimation

Phylogenetic relationships within *O. elongata* (including outgroups) were investigated based on Bayesian inference (see below for more details), and maximum parsimony (MP). The MP analysis was performed using PAUP* version 4.0b10 (Swofford, 2002) under the heuristic search option (tree-bisection-reconnection, branch swapping, random sequence addition, MaxTrees = 500) with 1000 random addition replicates.

A partitioned coalescent Bayesian analysis was performed with Beast 1.4.7 (Drummond and Rambaut, 2007) on the whole mtDNA dataset with the three regions represented as separate partitions (with specific rates of substitutions; see below) in the analysis and best-fit models as estimated by MrModeltest 2.3 (Nylander, 2004). Two independent runs of 60×10^6 generations were performed, sampling one tree every 1000 generations. Due to the lack of fossils for *Oreina* beetles, direct calibration of the tree topologies was not possible. Instead, branch lengths and node ages in the Beast analysis were estimated by applying a gene-specific mtDNA substitution rate based on a review of diverse arthropod taxa (Table 2). Average pairwise divergence rates were considered as follows: 1.73% My^{-1} for *COI*, 1.38% My^{-1} for *COII* and 0.61% My^{-1} for *16S*. Based on these values, a relaxed clock with log-normal branch length distribution was used and a Yule speciation model was applied to model population size through time (other prior parameters were set as default; Drummond et al., 2006). For each parameter, convergence of the independent runs was confirmed by the examination of their respective distributions in Tracer 1.4 (Rambaut and Drummond, 2007). After removing a burn-in period in each run, 95% divergence time confidence intervals were plotted on a majority-rule consensus tree (reconstructed using PAUP*; Swofford, 2002) using TreeAnnotator (Drummond and Rambaut, 2007).

2.5. Pairwise K-2P distances

To evaluate lineage divergence in light of the phylogenetic species concept (de Queiroz and Donoghue, 1988), pairwise

Table 2
Published substitution rates (pairwise divergence per million years) of three mtDNA gene regions for arthropods.

Gene region	Taxon	Substitution rate (% My^{-1})	Calibration (fossil or biogeographic)	Reference
COI	*Alpheus* (Decapoda: Alpheidae)	1.40	Uplift Isthmus of Panama (3 Mya)	Knowlton and Weigt (1998)
COI	*Sesarma/Sesarmoides* (Decapoda: Sesarmidae/Grapsidae)	1.66	Uplift Isthmus of Panama (3.1 Mya)	Schubart et al. (1998)
COI	*Ovobathysciola/Patriziella/Speonomus* (Coleoptera: Cholevidae)	2.50	Corsica-Sardinia plate separation (29 Mya)	Caccone and Sbordoni (2001)
COI	*Tetraopes* (Coleoptera: Cerambycidae)	1.50	Origin of habitats (1–20 Mya)	Farrell (2001)
COI	*Plateumaris* (Coleoptera: Chrysomelidae)	1.60	Fossils and biogeography (0.5–10 Mya)	Sota and Hayashi (2007)
16S	*Sesarma/Sesarmoides* (Decapoda: Sesarmidae/Grapsidae)	0.65	Uplift Isthmus of Panama (3.1 Mya)	Schubart et al. (1998)
16S	*Petrolisthes/Pachycheles* (Decapoda: Porcellanidae)	0.53	Uplift Isthmus of Panama (3 Mya)	Stillman and Reeb (2001)
16S	*Timarcha* (Coleoptera: Chrysomelidae)	0.45	Opening of Gibraltar strait (5.3 Mya)	Gomez-Zurita et al. (2000)
16S	*Iurus* (Scorpiones: Iuridae)	0.79	Separation Crete-Peloponnisos (5.33 Mya)	Parmakelis et al. (2006)
COII	*Timarcha* (Coleoptera: Chrysomelidae)	0.76	Opening of Gibraltar strait (5.3 Mya)	Gomez-Zurita et al. (2000)
COII	New Zealand cicadas (Hemiptera: Cicadidae)	2.00	Geological calibrations (9.3 Mya)	Arensburger et al. (2004)

Kimura-2P distances were calculated among specimens based on the *COI* dataset using the R package APE following Wiemers and Fiedler (2007) and Buerki et al. (2009). The *COI* region has proven to be a useful barcoding region for animals (Hebert et al., 2004) and might consequently provide valuable information to define taxa circumscription within the *O. elongata* complex.

2.6. Color pattern evolution

To track the evolution of color within *O. elongata* (i.e., based on the distribution of blue and green morphs), ancestral unordered parsimony reconstruction was performed on the majority-rule consensus tree from BEAST using "trace character history" implemented in MESQUITE, with the accelerated transformation optimization (ACCTRAN) parameter (Maddison and Maddison, 2009).

3. Results

3.1. Sequence dataset

Six outgroup and 64 ingroup specimens representing all seven described subspecies were collected from the 20 populations and amplified for the three mtDNA and the nuclear ITS2 region. Sequences are available at GenBank under Accession Nos. GQ220057–GQ220295.

When parsed through PAUP* (Swofford, 2002) only three polymorphic sites (in 589 bp) were identified at the ingroup level within the ITS2 region (amplification was successful for all specimens except four individuals from GLE). Two of those sites were restricted to one clade of the mtDNA phylogeny (the *O. e. siparii* clade, see below), whereas the remaining site corresponded to a split between taxa from eastern and western regions (separated by a line running to the west of MTT and FEN, Fig. 1). Based on this low level of polymorphism, the ITS2 region was not taken into consideration in the following analyses.

The concatenated mtDNA matrix included 1616 bp (including outgroups): 544 bp for *16S* (nine parsimony-informative [pi] sites among nine polymorphic sites in the ingroup), 397 bp for partial *COI* (43 pi sites among 47 polymorphic sites in the ingroup) and 675 bp for partial *COII* (13 pi sites among 25 polymorphic sites in the ingroup). The best-fit substitution models and parameter values suggested by MRMODELTEST (Nylander, 2004) are given in Table 3. Amplification failed in a few cases (for *COI*, single individuals from VAL, LAF, MTT, and three from PAS; for *COII*, two from PAS and AFR, and three from BOG, MTT, MLS, GLE and PDC; for *16S* all amplifications were successful). This missing data does not seem to have adversely affected the results, for these populations have not been pulled together in the phylogenetic analyses, nor do individuals with a locus missing fall away from other members of their population.

Table 3
Best supported models of molecular evolution and estimated parameter values for the analyzed genes.

Gene region	Model	Nucleotide frequencies					
		A	T	C	G	α	Tr/Tv
16S	HKY	0.40	0.36	0.15	0.09	–	3.517
COI	HKY + G	0.28	0.35	0.20	0.17	0.0158	3.708
COII	HKY + G	0.35	0.38	0.16	0.11	0.0996	4.330
ITS2	JC	0.26	0.28	0.22	0.24	–	–

α is the shape parameter for the gamma distribution and Tr/Tv is the transition–transversion ratio.

3.2. Phylogenetic analyses and divergence time estimation

Bayesian inference and MP analyses produced highly congruent topologies (with the same major nodes and branching order) and only the phylogenetic tree obtained with Bayesian inference in BEAST is shown (Fig. 2). The majority-rule consensus tree on which 95% confidence intervals for nodal ages are represented is based on 35,001 trees from two independent runs in BEAST. The applied burn-in period for each run was 25 million generations for the first run and 20 million generations for the second run.

Based on the average pairwise substitution rates for each marker, the analysis suggested that all the major clades within *O. elongata* were formed long before the last glacial period (with divergence spanning the last million years; Fig. 2). All basal nodes in this tree have very high support except one, with a Bayesian posterior probability (hereafter BPP) of 0.66 (clade encompassing subclades Vb, Vc and Vd; Fig. 2). Three of the subspecies are monophyletic (*O. e. styriaca*, *O. e. siparii* and *O. e. zangherii*) while the others are paraphyletic or polyphyletic. The most basal lineage contains individuals distributed in the central Alps (clade encompassing clades I and II; BPP: 0.99): clade I (BPP: 1.00) is composed of one population of *O. e. elongata*, whereas the other *O. e. elongata* population together with *O. e. ruffoi* form clade II (BPP: 0.84) (Figs. 1 and 2). The eastern Alpine population of *O. e. styriaca* (BPP: 1.00; clade III) and the central Apennines population of *O. e. siparii* (BPP: 1.00; clade IV) then split off as well-defined clades. Finally, the subspecies *O. e. occidentalis* (subclades Va and Vd, both distributed in the western Alps) is paraphyletic with respect to *O. e. zoiai* and *O. e. zangherii* (subclades Vb and Vc, respectively, both occurring in the northern Apennines). However, given the weak support (BPP: 0.66) of the node at the base of subclades Vb, Vc and Vd, one could also consider a trichotomy involving two *O. e. occidentalis* clades and *O. e. zangherii* with *O. e. zoiai*.

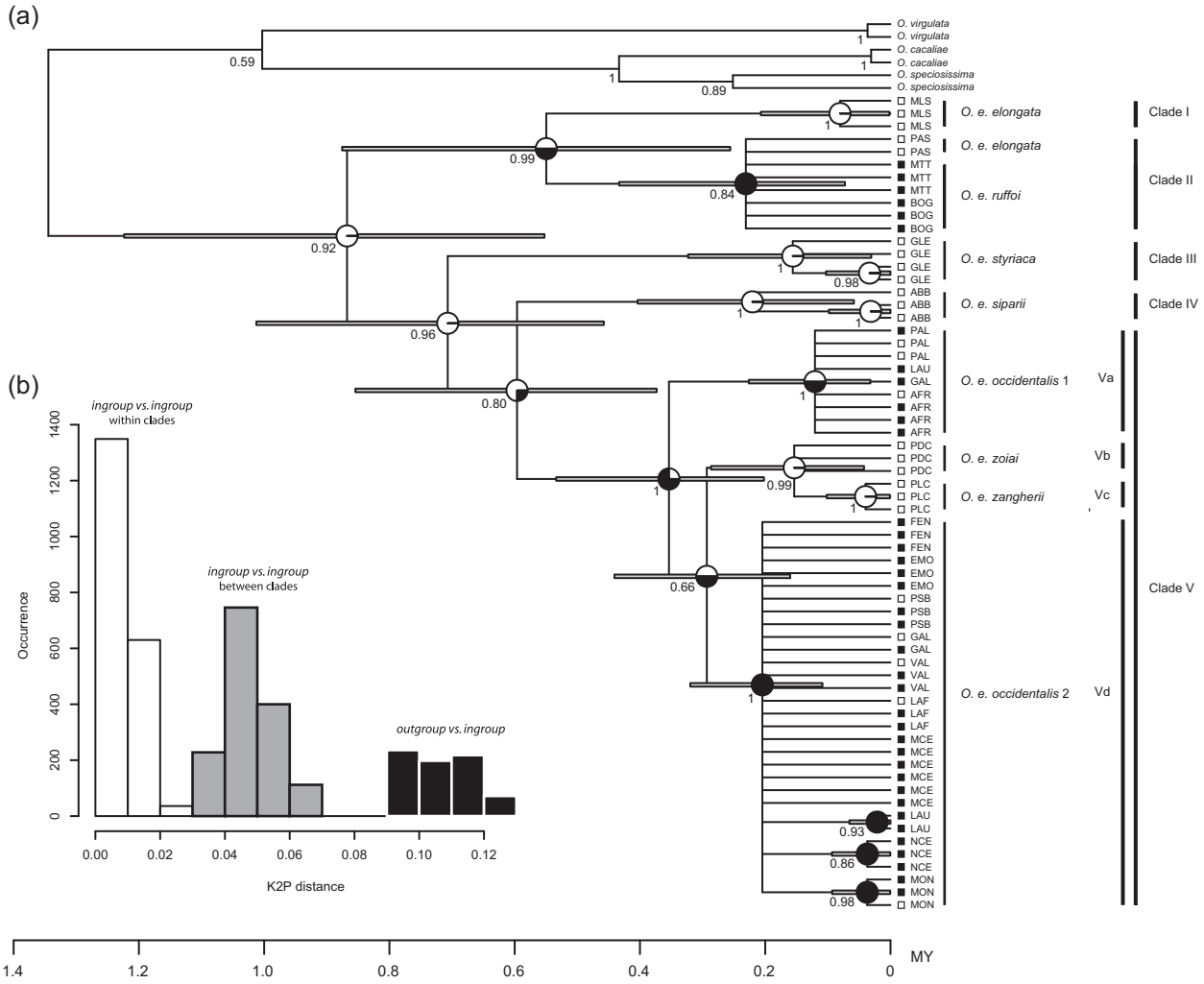

Fig. 2. (a) Majority-rule consensus tree from the MCMC stationary distribution of the partitioned coalescent Bayesian divergence time analysis, with median values and 95% confidence intervals for nodal ages. Pie-charts represent ancestral character evolution of color patterns reconstructed using the parsimony criterion in MESQUITE (blue morphs shown in black and green morphs in white). The color patterns of individuals are indicated by the squares. Values at the nodes are Bayesian posterior probabilities. Please see Fig. 1 and Table 1 for population codes. (b) Frequency distribution of intra-specific (both within and among clades) and inter-specific genetic divergence in *Oreina*. Four thousand and ninety-six intra-specific comparisons within *O. elongata* and 384 inter-specific pairs between *O. elongata* and outgroup taxa were calculated using Kimura's two parameter (K-2P) model.

3.3. Pairwise K-2P distances

The pairwise-distance histogram is multi-modal with three major modes (Fig. 2b): the first mode (distributed between zero and 0.03; in white on Fig. 2b) encompasses within-clade distances; the second mode (distributed between 0.03 and 0.07; in grey) includes only among-clade distances; the third major mode (distributed between 0.09 and 0.14; in black) comprises inter-specific distances including outgroup taxa.

3.4. Color pattern evolution

Despite the fact that there is strong geographical structure in the distribution of color variation (with green populations in the eastern part of the species range, and with blue and mixed populations in the west; Fig. 1), the pattern is less clear when mapped onto the phylogeny (Fig. 2). Blue and green morphs are present in the basal group (consisting of both *O. e. ruffoi* and *O. e. elongata* specimens; clade II), as well as in the two late-diverging clades comprising *O. e. occidentalis* (subclades Va and Vd). All other clades include only green morphs.

4. Discussion

Microsatellite data suggest very strong genetic differentiation of populations within *O. elongata* (with an overall F_{ST} of 0.381 and high pairwise values everywhere) and hence little migration (Margraf et al., 2007). This high level of isolation is confirmed here by the clustering of individuals from the same populations in the phylogeny and by the differentiation of the nuclear locus in *O. e. siparii*. Furthermore, the relatively low proportion of polymorphic sites for *16S* (1.65%) and *COII* (1.93%) is typical for this taxonomic (intra-specific level) and geographic scale (spanning 800 km in north–south and east–west directions) (Simon et al., 1994; Wiemers and Fiedler, 2007). However, the high level of polymorphism within the *COI* region widely used for animal barcoding (11.33%) and the bi-modal distribution in the K-2P distances at the intra-specific level (with inter-clades distances higher than 0.03) challenges the current species circumscription of *O. elongata*. Expectations from the phylogenetic species concept applied to *COI* (Wiemers and Fiedler, 2007) would suggest the split of these five clades into different species rather than subspecies, and hereafter, we favor the concept of a species complex when referring to *O. elongata*.

Within the *O. elongata* complex, the analyses strongly support the monophyly of three of the seven described subspecies: *O. e. styriaca* (clade III); *O. e. siparii* (clade IV); and *O. e. zangherii* (subclade Vc) (Fig. 2). The subspecies *O. e. elongata and O. e. ruffoi* are not monophyletic, while *O. e. occidentalis* is paraphyletic with respect to *O. e. zoiai* and *O. e. zangherii*, and *O. e. zoiai* is paraphyletic with respect to *O. e. zangherii* (Fig. 2).

In light of the unexpectedly high level of polymorphism at *COI* and the fact that four subspecies are not monophyletic, the taxon circumscription within *O. elongata* should be reviewed by increasing the sampling within populations and investigating additional morphological characters (e.g., the male genitalia and its inner structure). According to the present results, we would favor a solution encompassing five species, corresponding to the five defined clades (Fig. 2).

4.1. What does the phylogeography suggest about the evolution of color pattern in O. elongata?

The distribution of color pattern variation shows strong geographic structure, with green populations in the eastern Alps and Apennines, blue in southern Switzerland, and both blue and mixed populations scattered throughout the western Alps. However, this would be misleading if taken as a marker of large-scale phylogeographic structure, as shown by comparison with the mtDNA phylogenetic hypothesis. The three outgroup taxa are known to be polymorphic (Kippenberg, 1994), and both green and blue specimens are found in *O. elongata* clades II, Va and Vd. Nonetheless, all other clades including the putative most recent common ancestor (according to the MESQUITE analysis; Fig. 2) contain only green individuals, suggesting at least two or three independent origins of blue morphs as well as their loss in some populations.

Seven populations (AFR, GAL, LAF, MON, PAL, PSB, VAL) harbor the two morphs. This is unexpected because purifying selection by predators should lead to the loss of this diversity, and because population size is typically small, genetic drift would reinforce this effect. Two potential explanations for within-population polymorphism can be excluded, for it is clear that the two color morphs do not represent coexisting distinct species or subspecies, and neither is there a pattern that would suggest that polymorphism is maintained by current dispersal from monomorphic source populations. The evolutionary forces that maintain this unexpected color polymorphism in a chemically defended species therefore remain to be elucidated.

4.2. Did O. elongata, as a representative of the high altitude fauna, survive the cold periods of the Quaternary in situ in the Alps and Apennines?

The phylogenetic analysis and molecular clock hypothesis, even if only providing an approximate dating method, show that separation of the major clades in *O. elongata* occurred long before the last glacial maximum. In fact, divergence within the species spanned roughly half of the Quaternary, and the repeated glacial and interglacial periods may have been the engine for this differentiation. In contrast to lowland taxa, these beetles are likely to have occupied a larger area during cold periods, for although they may have been forced to retreat from the highest altitudes by the enlargement of glaciers within the Alps, a greater area of lower habitats would have become available. Despite this, our results suggest that the major lineages remained isolated during these periods. During warm spells like the present they display a reduced distribution, separated into more isolated, higher altitude populations. The general pattern in the phylogeography is that the populations from the central Swiss Alps and northern Italy are basal, with subsequent separation of the eastern, southern (Apennines) and western extremes of the distribution, and finally a second colonization of the Apennines. The basal populations in clades I and II in the Central Alps separate early and must have survived many glacial cycles in isolation from the rest of the species, perhaps in refuges within the Alps. The ancestors of the other clades remained in contact elsewhere, and may have inhabited an expanded range during repeated cold periods south of the Alps across northern Italy and the northern part of the Adriatic (which would have been exposed at such times by falling sea levels). Populations from this region seem to have given rise to the subspecies *O. e. styriaca* in the eastern Alps, and *O. e. siparii* in the central Apennines between 0.7 and 0.6 Mya. The remaining populations may have survived colder periods along the edge of the western Alps. Geographical structuring within subspecies *O. e. occidentalis* (with subclades Va and Vd diverging c. 0.4 Mya) is associated with the north–south orientated Guisane and Durance valleys, with the populations PAL and AFR found to the south and in the north the populations LAU and GAL (both containing individuals from subclades Va and Vd, suggesting some gene flow). The remaining populations form subclade Vd. Subclades Vb and Vc are associated with a second invasion into the Apennines around 0.3 Mya (corresponding to subspecies *O. e. zoiai* and *O. e. zangherii*, respectively).

The structuring within *O. elongata* is very similar to that seen in Alpine plants. The contemporary distributions of alpine populations correspond to the four refugia proposed for alpine plants along the southwestern, southern and eastern edges of the Alps (Schönswetter et al., 2005; Margraf et al., 2007). Peripheral refugia in the southwestern and southern Alps have also been suggested for the mountain ringlet, *Erebia epiphron* (Schmitt et al., 2006), and the caddisfly, *Drusus discolor* (Pauls et al., 2006). *O. elongata* therefore joins the list of high altitude plant and animal species that appear to have survived glacial periods close to their current distribution (Schmitt, 2009). The phylogeography presented here indicates that divergence of the major clades occurred between 1 and 0.3 million years ago, suggesting that *O. elongata* survived the Quaternary within or at the edge of the Alps and Apennines.

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